PATENT SPECIFICATION

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COMPLETE SPECIFICATION

Improvements in or relating to Oestrogenic Substances

We, NATIONAL RESEARCH DEVELOPMENT CORPORATION, a British Corporation, of 1, Tilney Street, London, W.1, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to the isolation of naturally-occurring oestogenic substances and is particularly concerned with the isolation of an oestrogen from a Siamese plant which was formerly incorporated in a Siamese rejuvenating drug.

This drug, which was reported in 1932 and 1933 to have been used in Siam, consisted of the tuberous roots of a plant, powdered and mixed with honey and myrobalans. It was reported to have brought on menstruation in old women, to have enabled an impotent old man to become the father of new offspring and also in some cases to have been toxic. The tuberous root which had these effects was thought to be that of *Butea superba*, Roxb.

Schering-Kahlbaum obtained supplies of the roots and in Specifications Nos. 437,051 and 453,583 they describe extraction methods using water, acetone, chloroform and methanol. All the extracts were highly oestrogenic in the rat but that prepared with water was the most potent. The specification also described various ways of separating the oestrogen from inactive matter as follows:—

(a) The roots were extracted with hot water; the extract filtered and the filtrate evaporated to a syrup, which was then diluted with a large excess of methanol and filtered. The filtrate possessed oestrogenic activity. Repetition of this process concentrated the active matter further.

(b) The active matter from (a) obtained by evaporating the filtrate to dryness was re-dissolved in water and precipitated with ammonium sulphate. Oestrogenic activity was found in the precipitate.

(c) Dried powdered roots were extracted with methanol. The methanol solution was evaporated; the residue dissolved in water and precipitated with ammonium sulphate. The precipitate was partitioned between benzene and 70% ethanol and the oestrogenic activity was then found mainly in the lower layer.

(d) The precipitated matter from (b) possessing activity was re-dissolved in methanol and ether added to precipitate impurities; this being repeated twice, the oestrogen remaining largely in solution.

(e) The active matter from (d) obtained by evaporating the solution to dryness was dissolved in ethanol and water added until the solution contained 70% of ethanol. This was then extracted with benzene. The oestrogen remained in the aqueous ethanol.

Schoeller, Dohrn and Hohlweg (United States Specification No. 2,112,712) again found the roots to be rich in oestrogen (highly active orally as well as subcutaneously). They extracted the dried, powdered roots with ethanol and then filtered and evaporated the solution. They note the isolation of an oestrogen of the empirical formula $C_{19}H_{22}O_6$.

Later these workers published their work in short form (Naturwissenschaften 28, 532 (1940)). They described the acquisition of the oestrogenic roots from Siam, which they call *butea superba*, but they noted that the oestrogenic plant appeared to be different from the *butea superba* growing in botanical gardens in Ceylon. They estimated the oestrogenic activity of the roots to be 180,000 Rat Units per kilogram or equivalent to 150 mg. of oestrone per kg. They prepared highly oestrogenic concentrates from which Butenandt and Jacobi at Danzig, first isolated the oestrogen and the gave the empirical formula $C_{19}H_{20}O_6$ (Butenandt, Naturwissenschaften 28, 533 (1940)). Butenandt found that the compound reduced Fehling's solution, was destroyed by alkali, gave an anhydro derivative $C_{19}H_{20}O_5$ with HCl and gave a monomethyl ether.

Schoeller, Dohrn and Hohlweg (Naturwissenschaften 28, 532 (1940)) also described

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the various physiological effects on the rat of the new compound. These are typical of stimulation by oestrogen. The new substance lay between oestradiol and oestrone in activity when assayed by the subcutaneous route in the Allen-Doisy test; it was much more active orally than either oestradiol or oestrone.

It was finally established that the plant producing the tuberous roots containing the oestrogen is of a new species which is named *pueraria mirifica*, Airy Shaw et Suvatabandhu (Kew Bulletin (1952, 549)).

It is an object of this invention to provide an improved process for the isolation of the aforesaid oestrogen to which we have ascribed the empirical formula $C_{20}H_{22}O_6$, but which is presumably identical with that described by Butenandt to which he gave the empirical formula $C_{19}H_{20}O_6$. This compound will hereinafter be referred to as "miroestrol". Particulars of the ultra-violet and infra-red absorption spectra are given below in Example 1.

According to the process of the present invention for the production of miroestrol, a methanol or ethanol extract of tuberous root of *pueraria mirifica* is subjected to partition chromatography.

Two methods were used for assaying the various extracts and fractions for oestrogen content; bioassay by the mouse uterine weight assay as used by Pope & Roy (Biochem. J. 53, 427 (1953)) and paper chromatographic assay. Bioassay which can estimate the total oestrogenic activity of a crude extract was used during the development of the isolation process but once miroestrol had been concentrated sufficiently it was possible and convenient to estimate it by paper chramatography.

According to a preferred embodiment of the invention powdered tuberous roots of *pueraria mirifica* are extracted with aqueous methanol or ethanol and then with pure methanol or ethanol and the solutions separated, for example by filtration, combined together and evaporated to dryness. The residue from this is fractionated by solution in methanol and ether added to precipitate impurities. Most of the oestrogen remains in solution and is further purified by partition in a solvent system containing water, methanol, ethyl acetate and benzene. The active material remains in the lower phase and is next subjected to partition chromatography.

Preferably the partition chromatography is carried out on a column of kieselguhr and preferably also the mobile phase is a mixture of benzene and ethyl acetate and the stationary phase is aqueous methanol.

The following examples illustrate the invention:—

1. The initial material is tuberous roots of *pueraria mirifica* sliced, sun-dried and powdered to a fine powder in a hammer-mill and having a dry matter content of approximately 87%.

5 kg of powder was boiled and stirred for 0.5 hour with a mixture of 11 litres of methanol and 1 litre of water in a 20 litre flask, provided with a reflux condenser and a stirrer consisting of a stainless steel shaft and six small vanes set at an angle of 45° to the shaft. The stirrer was driven by a 0.08 H.P. electric motor at 1350 r.p.m. by direct coupling with rubber tubing. The solution was separated by filtration through an 8-inch Buchner funnel having a tin-plate, cylindrical extension piece to increase its capacity, the filtrate passing directly into a 20 litre distillation flask. The plant residue was then similarly extracted a second time with 10 litres of methanol and a third time also with 10 litres of methanol. The combined filtrates were evaporated to dryness under reduced pressure, the residue shaken with 3 litres of methanol and left to stand for 18 hours after which the granular precipitate was filtered off and the filtrate evaporated to dryness at reduced pressure. The residue was then dissolved in 1 liitre of methanol and 4 litres of ether added. On leaving overnight a gummy precipitate formed from which the supernatant solution was decanted. This solution was then evaporated to dryness and the residue dissolvel in 1 litre of methanol and 4 litres of ether added. Again after allowing to stand the clear supernatant solution was decanted off, the precipitate being redissolved in 500 ml of methanol and re-precipitated with 2 litres of ether. The combined supernatant solutions were then evaported to dryness at reduced pressure. This residue was shaken with 2.5 litres of ethyl acetate at room temperature and the solution left to clarify. The filtrate was evaporated to dryness and partitioned between:—

Benzene - - -	2500 ml
Ethyl acetate - -	250 ml
Methanol - - -	1250 ml
Water - - - -	1250 ml

The lower phase was separated off and the upper phase washed with 1250 ml of 50% aqueous methanol. The combined lower phases were evaporated to dryness at reduced pressure and the residue chromatographed on a large column using the partition method. The solvent system used was:—

Benzene - - -	8000 ml
Ethyl acetate - -	3000 ml
Methanol - - -	5000 ml
Water - - - -	5000 ml

The column consisted of a 6 ft. length of glass pipeline of 4 inches internal diameter with a suitable reduction piece and stop-cock at its lower end. It was packed with kieselguhr (Celite 545, supplied by Johns-Manville Co., Ltd., London, the word "Celite" being a registered Trade Mark) by the method of Martin (Biochem. Soc. Symposia No. 3, p. 11, 1949). The kieselguhr was slurried with solvent by mechanical stirring in a flask and the slurry transferred by pressure into the top of

the column. Packing was done with a stainless steel ramrod having a perforated head ($\frac{1}{8}$ inch diameter holes) and a sectional shaft. The height of the kieselguhr was then 28 inches. The residue to be chromatographed was added in the minimum quantities of a mixture of stationary and mobile phases necessary to effect solution. The partition column was operated in the usual manner and suitable aliquots of the various fractions examined by paper chromatography. The chromatograms were sprayed with a suitable visualising agent such as diazotised *p*-aminophenyl-2-diethylaminoethyl sulphone, methanolic potassium hydroxide or periodic acid.

The fractions from the 4-inch diameter column containing miroestrol were then rechromatographed on a 2-inch diameter kieselguhr column using the same solvent system. The fractions containing miroestrol were again identified by paper chromatography and were collected together and run similarly on a second 2-inch column. The fractions from this column which contained miroesterol crystallised partially and were washed with ethyl acetate yielding crystals (140 mg). Recrystallisation from methanol gave pure miroestrol (120 mg), m.p. (with decomposition) 270° C., optical rotation $[\alpha]_D^{27°} = +301°$.

The ultra-violet absorption of miroesterol in ethanol solution was as follows:—

ethanol
$\lambda_{max.}$ 220 mμ;

ethanol
$\lambda_{max.}$ 287 mμ $\varepsilon = 3480$

The infra-red absorption spectrum of miroesterol (crystalline state; as paraffin paste) showed absorption peaks at:—
3334 cm.$^{-1}$, 2865 cm.$^{-1}$, 1712 cm.$^{-1}$, 1664 cm.$^{-1}$, 1621 cm.$^{-1}$, 1597 cm.$^{-1}$, 1508—1504 cm.$^{-1}$, 1460—1453 cm.$^{-1}$, 1401 cm.$^{-1}$, 1362 cm.$^{-1}$, 1325 cm.$^{-1}$, 1282 cm.$^{-1}$, 1245—1242 cm.$^{-1}$, 1224—1218 cm.$^{-1}$, 1183 cm.$^{-1}$, 1176 cm.$^{-1}$, 1170 cm.$^{-1}$, 1160 cm.$^{-1}$, 1155 cm.$^{-1}$.

The oestrogenic activity of miroestrol was about 1.3 times that of oestradiol-17β in the mouse uterine weight test and about one-fourth that of oestradiol-17β in the rat vaginal smear test (Allen & Doisy, Amer. J. Physiol. 69, 577 (1924)).

It was estimated by mouse bioassay methods that the overall recovery of miroestrol from the plant source was greater than 50%.

2. Example 1 was repeated using an equal volume of ethanol instead of methanol for the two extractions of the plant.

What we claim is:—

1. A process for the production of miroestrol as herein defined wherein a methanol or ethanol extract of tuberous roots of *pueraria mirifica* is subjected to partition chromatography.

2. A process for the production of a miroestrol wherein powdered tuberous roots of *pueraria mirifica* are extracted with aqueous methanol or ethanol, the extract is separated from the roots and then the extracted roots are extracted with pure methanol or ethanol, the extract is separated from the roots, the extracts are combined together and evaporated to dryness, the residue is fractionated by solution in methanol and addition of ether to precipitate impurities, the residue obtained by evaporation to dryness of the supernatant solution is purified by partition in a solvent system containing water, methanol, ethyl acetate and benzene and the lower phase is subjected to partition chromatography.

3. A process as claimed in Claim 1 or 2 wherein the partition chromatography is carried out on a column of kieselguhr.

4. A process as claimed in Claim 3 wherein the mobile phase is a mixture of benzene and ethyl acetate and the stationary phase is aqueous methanol.

5. A process for the production of miroestrol substantially as described with reference to either of the examples.

6. Miroestrol when produced by the process claimed in any one of the preceding claims.

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PROVISIONAL SPECIFICATION

Improvements in or relating to Oestrogenic Substances

We, NATIONAL RESEARCH DEVELOPMENT CORPORATION, a British Corporation, of 1, Tilney Street, London, W.1, do hereby declare this invention to be described in the following statement:—

This invention relates to the isolation of naturally-occurring oestrogenic substances and is particularly concerned with the isolation of an oestrogen from a Siamese plant which was formerly incorporated in a Siamese rejuvenating drug.

This drug, which was reported in 1932 and 1933 to have been used in Siam, consisted of the tuberous roots of a plant, powdered and mixed with honey and myrobalans. It was reported to have brought on menstruation in old women, to have enabled an impotent old man to become the father of a new offspring and also in some cases to have been toxic. The tuberous root which had these effects was thought to be that of *Butea superba*, Roxb.

Scherin-Kahlbaum obtained supplies of the

roots and in French Specification No. 782,375 they describe extraction methods using water, acetone, chloroform and methanol. All the extracts were highly oestrogenic in the rat but that prepared with water was the most potent. The specification also describes various ways of separating the oestrogen from inactive matter as follows:—

(a) The roots were extracted with hot water; the extract filtered and the filtrate evaporated to a syrup, which was then diluted with a large excess of methanol and filtered. The filtrate contained the activity. Repetition of this process concentrated the activity further.

(b) The active matter from (a) was re-dissolved in water and precipitated with ammonium sulphate. The activity was found in the precipitate.

(c) Dried powdered roots were extracted with methanol. The methanol solution was evaporated; the residue dissolved in water and precipitated with ammonium sulphate. The precipitate was partitioned between benzene and 70% ethanol and the oestrogenic activity was then found mainly in the lower layer.

(d) The precipitated matter from (b) containing activity was re-dissolved in methanol and precipitated with ether; this being repeated twice, the oestrogen remaining largely in solution.

(e) The active matter from (d) was dissolved in ethanol and water added until the solution contained 70% of ethanol. This was then extracted with benzene. The oestrogen remained in the aqueous ethanol.

Schoeller, Dohrn and Hohlweg (United States Specification No. 2,112,712) again found the roots to be rich in oestrogen (highly active orally as well as subcutaneously). They extracted the dried, powdered roots with ethanol and then filtered and evaporated the solution. They note the isolation of an oestrogen of the empirical formula $C_{19}H_{22}O_6$.

Later these workers published their work in short form (Naturwissenschaften 28, 532 (1940)). They described the acquisition of the oestrogenic roots from Siam, which they call *butea superba*, but they noted that the oestrogenic plant appeared to be different from the *butea superba* growing in botanical gardens in Ceylon. They estimated the oestrogenic activity of the roots to be 180,000 Rat Units per kilogram or equivalent to 150 mg. of oestrone per kg. They prepared highly oestrogenic concentrates which were given to Butenandt and Jacobi at Danzig, who first isolated the oestrogen and gave the empirical formula $C_{19}H_{20}O_6$ (Butenandt, Naturwissenschaften 28, 533 (1940)). Butenandt found that the compound reduced Fehling's solution, was destroyed by alkali, gave an anhydro derivative $C_{19}H_{20}O_5$ with HCl and gave a monomethyl ether.

Schoeller, Dohrn and Hohlweg (Naturwissenschaften 28, 532 (1940)) also described the various physiological effects on the rat of the new compound. These are typical of stimulation by oestrogen. The new substance lay between oestradiol and oestrone in activity when assayed by the subcutaneous route in the Allen-Doisy test; it was much more active orally than either oestradiol or oestrone.

It was finally established that the plant producing the tuberous roots containing the oestrogen is of a new species which is named *pueraria mirifica*, Airy Shaw et Suvatabandhu (Kew Bulletin (1952, 549)).

It is an object of this invention to provide an improved process for the isolation of the aforesaid oestrogen which has been found to have the empirical formula $C_{19}H_{22}O_6$ but which is apparently identical with the compound described by Butenandt to which he gave the empirical formula $C_{19}H_{20}O_6$. This compound will hereinafter be referred to as "miroestrol".

According to the process of the present invention for the production of miroestrol, a methanol extract of tuberous roots of *pueraria mirifica* is subjected to partition chromatography.

According to a preferred embodiment of the invention powdered roots of *pueraria mirifica* are extracted with methanol and then with aqueous methanol and the solution separated by filtration and evaporated to dryness. The residue from this is fractionated by solution in methanol and precipitation with ether. Most of the oestrogen remains in solution and is further purified by partition in the solvent system water, methanol, ethyl acetate, benzene. The active material remains in the lower phase and is next subjected to partition chromatography.

Preferably the partition chromatography is carried out on a column of kieselguhr and preferably also the mobile phase is a mixture of benzene and ethyl acetate and the stationary phase is aqueous methanol.

The following example illustrates the invention:—

The initial material was powdered roots of *pueraria mirifica* having a dry matter content of 87%.

500 g. of powdered roots was boiled for 0.5 hour with 2.5 litres of methanol and the solution separated by filtration. The powder was then re-extracted with 2.5 litres of boiling methanol-water (80:20 v/v) and this solution separated by filtration. The latter process was then repeated. The combined solutions were then evaporated to dryness at reduced pressure and the residue mixed with 500 ml. of methanol. The process was then repeated on a second 500 g. quantity and the two final 500 ml. methanol solutions combined, filtered and the filtrate evaporated at reduced pressure to 500 ml. To this 500 ml. of ether was added slowly with shaking. A gummy precipitate formed from which the solution was decanted. The solution was

evaporated to dryness and the residue remaining dissolved in methanol (120 ml.) and shaken while ether (500 ml.) was added. Again the precipitate and solution were separated by decantation. This precipitate was re-treated by dissolving in methanol (100 ml.) and reprecipitating with ether (300 ml.). The two solutions were then combined, evaporated to dryness and treated with ethyl acetate (500 ml.). The solution was evaporated to dryness and the residue equilibrated in the solvents benzene (500 ml.), ethyl acetate (50 ml.), methanol (250 ml.) and water (250 ml). The upper phase of this was extracted with fresh lower phase (250 ml.) and the two lower phase solutions combined and evaporated to dryness. The resulting final extract was found to contain much of the original oestrogenic activity.

Another 5000 g. of the initial material was then processed similarly and all the final extracts (from 6000 g.) bulked. This extract, about 30 g. in all, had approximately 50% of the original oestrogenic activity, or about 100 mg. of oestrogen expressed as miroestrol.

The 30 g. of extract was then divided into eight equal portions. Each portion was chromatographed on a column using the partition method. The column consisted of a vertical glass tube 5 cm. in diameter and 100 cm. long with a glass tap at its lower end. A plug of glass wool was first pressed tightly into the column which was then packed with kieselguhr ("Celite 545") in the manner described by Martin (Biochem. Soc. Symposia No. 3, p. 11, 1949) using the system benzene (800 ml.), ethyl acetate (300 ml.), methanol (500 ml.), water (500 ml.). The top phase was then run through the column until its top surface was level with the top of the packing. The column of kieselguhr (from 400 g. dry weight) when completed was 62 cm. tall. The material to be chromatographed (approximately 3.6 g.) was then dissolved in 2 to 3 ml. of lower phase and the solution placed on to the surface of the packing. Elution with mobile phase was then begun and the following volumes of elute collected in successive fractions: 1000, 500, 250, 250, 250, 250, 500, 600 ml. Finally residual plant extract was removed from the column by eluting with methanol. Data for a typical chromatogram are in Table I.

TABLE I

CHROMATOGRAM OF 3.7 G. EXTRACT (DERIVED FROM 652 G. DRY MATTER OF PUERARIA TUBEROUS ROOT)

No. of fraction	Volume of successive fractions eluted (ml.)	Wts. of plant extract in fraction (mg.)	Oestrogenic activity calculated as miroesterol (mg.)
1	1000	1662	nil
2	500	344	nil
3	250	59	nil
4	250	56	0.15
5	250	55	2.5
6	250	42	>8
7	500	55	>2
8	600	59	nil
9	methanol eluate	1309	nil
		3641	>12.65

These results show that the activity after one chromatographic process has been concentrated into approximately 150 mg. of material (25-fold concentration). Activity recovered is at least 50% of that originally in the plant material.

Active fractions from all eight chromatograms, on which were processed the extract from 6000 g. of initial material, were pooled and re-processed through two more chromatograms. Finally 82 mg. of crystalline matter, sparingly soluble in ethyl acetate, was obtained. Recrystallisation of this yielded 47.5 mg. of microscopic, colourless, rod-like crystals, m.p. (with decomposition) 275° C., which were characterised as miroestrol by paper chromatography using three solvent systems.

The product has the empirical formula $C_{19}H_{22}O_6.H_2O$.

The substance was soluble in alcohols but very sparingly soluble in ether, ethyl acetate, benzene or chloroform. It formed a penta-acetate $C_{19}H_{17}O(OAc)_5$. It had activity equal to that of oestradiol in the mouse uterine weight test and one-fourth that of oestradiol in the Allen-Doisy test in the rat, when given subcutaneously in each case.

The oestrogenic activity of extracts was estimated by the mouse uterine weight method. No attempt was made to use the method in its statistically valid form although at least two dose levels of each unknown extract were used and the responses compared with those given by known quantities of the standard oestrogen, oestradiol. This enabled estimates of the oestrogenic activity of extracts to be made that were in practice sufficiently accurate to enable the work of isolation of the oestrogen to pro-

ceed smoothly. A description of the bioassay as used in this way is given by Pope and Roy, Biochem. J. 53, 427 (1953).

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